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Abstract: The early life period is characterized by fast growth and development, which can lead to high reactive oxygen species (ROS) production. Young animals thus have to balance their investment in growth versus ROS defence, and this balance is likely mediated by resource availability. Consequently resources transferred prenatally by the mother and nutritional conditions experienced shortly after birth may crucially determine the oxidative status of young animals. Here, we experimentally investigated the relative importance of pre- and early postnatal nutritional conditions on the oxidative status of great tit nestlings (Parus major). We show that resources transferred by the mother through the egg and nutritional conditions encountered after hatching affect the oxidative status of nestling in a sex-specific way. Daughters of non-supplemented mothers and daughters which did not receive extra food during the early postnatal period had higher oxidative damage than sons, while no differences between sons and daughters were found when extra food was provided pre- or postnatally. No effect of the food supplementations on growth, fledging mass or tarsus length was observed, indicating that female nestlings maintained their investment in growth at the expense of ROS defence mechanisms when resources were limited. The lower priority of the antioxidant defence system for female nestlings was also evidenced by lower levels of specific antioxidant components. These results highlight the important role of early parental effects in shaping oxidative stress in the offspring, and show that the sensitivity to these parental effects is sex-specific.

DOI: https://doi.org/10.1007/s00442-014-3100-x

Sex-specific effects of prenatal and postnatal nutritional conditions on the oxidative status of great tit nestlings

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Short title: Sex-specific oxidative status

Author Contributions: M.G. and B.T. conceived and designed the experiment. M.G. conducted the fieldwork and analyzed the data. M.G. and D.C. did the laboratory work. M.G. and B.T. wrote the manuscript.

Abstract

The early life period is characterized by fast growth and development, which can lead to high reactive oxygen species (ROS) production. Young animals thus have to balance their investment in growth versus ROS defence, and this balance is likely mediated by resource availability. Consequently resources transferred prenatally by the mother and nutritional conditions experienced shortly after birth may crucially determine the oxidative status of young animals.

Here we experimentally investigated the relative importance of pre- and early postnatal nutritional conditions on the oxidative status of great tit nestlings (*Parus major*). We show that resources transferred by the mother through the egg and nutritional conditions encountered after hatching affect the oxidative status of nestling in a sex-specific way. Daughters of non-supplemented mothers, and daughters, which did not receive extra food during the early postnatal period had higher oxidative damage than sons, while no differences between sons and daughters were found when extra food was provided pre- or postnatally. No effect of the food supplementations on growth, fledging mass or tarsus length was observed, indicating that female nestlings maintained their investment in growth at the expense of ROS defence mechanisms when resources were limited. The lower priority of the antioxidant defence system for female nestlings was also evident by lower levels of specific antioxidant components. These results highlight the important role of early parental effects in shaping oxidative stress in the offspring, and show that the sensitivity to these parental effects is sex-specific.

Keywords: early development; ecophysiology; food availability; oxidative stress; maternal effects

Introduction

Already during the first stages of life, before and shortly after birth, individuals differ in the quantity and quality of resources they have available, either because of environmental variation in resource abundance and / or different maternal investment strategies (Mousseau and Fox 1998; Christians 2002). These differences in early nutritional conditions can have important and long-lasting effects on an individual's physiology, morphology and life history (Mousseau and Fox 1998; Lindström 1999; Monaghan 2008).

The prenatal and early postnatal periods are characterized by fast growth and development, and are therefore nutritionally highly demanding (Keller and Van Noordwijk 1994; Naef-Daenzer and Keller 1999). At the same time, fast growth and development result in increased production of free radicals (Rollo et al. 1996), which may negatively affect fitness, both in the short- and the long-term (Costantini 2008; Metcalfe and Alonso-Alvarez 2010). Individuals thus face trade-offs and constraints in their investment in growth and the free radical scavenging system early in life (Alonso-Alvarez et al. 2007; Hall et al. 2010), and these trade-offs are likely mediated by food availability (Catoni et al. 2008a; Monaghan et al. 2009).

One consequence of high free radical production is oxidative stress, which is commonly defined as an unbalance between the production of reactive oxygen species (ROS) and antioxidant responses, in favor of the former, leading to an increase in oxidative damage (Sies 1991; Halliwell and Gutteridge 2007). ROS are constantly produced in the mitochondria as byproducts of cell respiration (Handy and Loscalzo 2012) and above certain concentrations, they become harmful and can cause cellular damage (Halliwell and Gutteridge 2007).

Organisms have evolved a complex antioxidant machinery to counter the toxicity of ROS and maintain normal physiological conditions in the cell (Halliwell and Gutteridge 2007; Pamplona and Costantini 2011). The first line of defense includes scavengers, such as thiols groups, which directly react with and neutralize oxidizing agents (Dickinson and Forman 2002; Cremers and Jakob 2013). A second line of defense includes enzymes, such as glutathione peroxidase, which catalyze the reduction of dangerous oxidizing agents (Arthur 2000).

Evidence is accumulating that the ability to mitigate the negative effects of ROS activity is an important factor underlying variation in individual fitness (Costantini 2008; Metcalfe and Alonso-Alvarez 2010). Antioxidant capacity is positively, while oxidative damage negatively related to survival (Freeman-Gallant et al. 2011; Saino et al. 2011). Furthermore recent studies showed that the ability of nestlings to cope with oxidative stress predicts their fledging success (Losdat et al. 2013) and their recruitment probability (Noguera et al. 2012), highlighting that an efficient antioxidant protection is beneficial from the first stages of life. However, investment in such an efficient oxidative protection is likely associated with costs.

Many components of the antioxidant machinery (e.g. carotenoids, vitamin E, vitamin C) are acquired directly through the food (Catoni et al. 2008a; Catoni et al. 2008b). Moreover dietary components with no specific antioxidant properties are used to build important antioxidant molecules (e.g. thiols and enzymes) (Dringen et al. 1999; Lee et al. 2013). Studies on laboratory and wild animals showed that differences in the protein content of the diet can affect the oxidative status of an individual (Feoli et al. 2006; Cohen et al. 2009; Beaulieu et al. 2010). In rats, for example, protein deficiency gave rise to higher oxidative damage and decreased antioxidant activity (Bonatto et al. 2006; Feoli et al. 2006), whereas cysteine-rich food

reduced oxidative stress and increased thiols levels (Blouet et al. 2007). Furthermore a high protein content in the diet improved the antioxidant protection in juvenile white-throated sparrows (*Zonotrichia albicollis*) (Alan and McWilliams 2013). These findings highlight the crucial role of high protein intake for the proper functioning of the antioxidant machinery.

Early in life, individuals depend mainly on maternal resources transferred prenatally (e.g. into the egg) and (in altricial species) on resources provided by the parents after birth for their protection against oxidative damage, and they start to develop their own protective machinery only after a few weeks of life (Karadas et al. 2005; Surai and Fisinin 2013). Consequently food availability encountered by the mother during egg laying and food availability encountered by the parents during the rearing period are expected to affect the offspring's antioxidant machinery. Accordingly, offspring are predicted to have highest oxidative protection and lowest oxidative damage when nutritional conditions are favorable both before and after birth.

However, higher pre- and early postnatal food availability might also trigger faster growth, which may create extra ROS. Under this scenario, favorable pre- and early postnatal food conditions could lead to higher, rather than lower oxidative stress. Thus, it is important to assess the effect and relative importance of pre- and postnatal nutritional conditions on antioxidant status and growth, respectively. Here we experimentally manipulated food availability during egg laying and food availability after hatching in a 2 x 2 design in a natural population of great tits (*Parus major*), in order to test how pre- and early postnatal conditions affect oxidative status and growth of male and female offspring.

Material and Methods

Study species and experimental protocol

The study was conducted between March and June 2012, in a nestbox-breeding population of great tits in Zurichbergwald, a forest close to Zurich, Switzerland (47°20'08'' N, 8°30'01'' E). A total of 350 nestboxes (12.5cm x 12.5cm x 26.5 cm, Type Varia), placed at a distance of 100-150 meters to one another along forest roads, were regularly visited from the beginning of the breeding season onwards in order to monitor the progress of nest building and the start of egg laying. At an advanced stage of nest building, but before the first egg was laid (mean ± SD: 7.1 ± 4.7 days before the first egg was laid), we experimentally manipulated the food availability of the breeding pairs by providing additional food in half of the nestboxes. To this end, we attached a small plastic cup on the inside wall of all nestboxes. Placing the cup inside the nestbox, not visible from the outside, ensured that only the breeding pair consumed the food we provided. We randomly assigned nestboxes to the pre-laying food supplementation (pre-F) or to the control group (pre-NF). Pre-F nests received a food supplementation of 15g of maggots (*Sarcophaga* spp.), which contain high levels of proteins and lipids (Hwangbo et al. 2009; Jensen et al. 2011), every other day. All maggots were eaten within two days (M.G. pers. obs.). We cannot exclude that males ate a part of the supplemented food. However, the significant effect of the pre-laying food supplementation on offspring traits (see Results) shows that the pre-laying treatment was effective in influencing maternal egg provisioning. Indeed, food-supplemented females tended to lay heavier eggs than non-supplemented females ($F_{1,113} = 3.553$, $P = 0.062$). No difference in clutch size between supplemented and non-supplemented females was observed ($F_{1,126} = 0.883$, $P = 0.349$). Given that nestlings were cross-fostered between nests, we can exclude the possibility that the

effects of the pre-laying treatment on offspring traits were due to carry-over effects of the pre-laying treatment on parental provisioning after hatching. The pre-NF nests were visited and treated as the pre-F nests, but no food was added to their plastic cup. After the clutch was completed, the food supplementation stopped and the females incubated their eggs without receiving extra food.

To manipulate the food availability experienced by the nestlings after hatching and to be able to disentangle prenatal and postnatal effects on offspring oxidative status, we carried out a complete cross-fostering (i.e. a brood swap) one day after hatching between nests with the same hatching date and a similar brood size (N = 64 dyads). Cross-fosterings were alternatingly performed between nests with the same and a different pre-laying treatment. During the transport between nests, nestlings were kept warm in a padded box to minimize potential stress. After cross-fostering, one brood of each dyad was randomly assigned to the post-hatching food supplementation group (post-F, N = 64 broods), whereas the other received no extra food during the nestling period (post-NF, N = 64 broods). The post-hatching food supplementation followed the same protocol as the pre-laying treatment. Post-F nests received 15g of maggots (*Sarcophaga* spp.) every other day from day 1 until day 13 post-hatching. The post-NF broods were visited and treated as the post-F broods, but no food was added to their plastic cup.

Nestling measurements

We measured nestling body mass on day 1 post-hatching (N = 756 nestlings) (hereafter 'hatching mass'), on day 3 (N = 728 nestlings), on day 9 (N = 661 nestlings) and on day 15 (hereafter 'fledging mass'), shortly before fledging (N = 517 nestlings). On day 15, we also measured tarsus length, a proxy for body size. We

determined the growth rate during the linear growth period (day 3 to day 9) by calculating [(Ln (body mass day 9) – (Ln (body mass day 3)]/ 6.

Nestlings were marked individually on day 1 by clipping down feathers on their head and back. When nestlings were 9 days old, they were ringed with a numbered aluminium ring and a blood sample was collected from the tarsal vein for the analysis of oxidative stress markers and molecular sex determination (as described in Tschirren et al. 2003). The blood was kept cool until it was centrifuged (10'621x g for 10 minutes; Eppendorf 5417C centrifuge) on the same day. After centrifugation, plasma and red blood cells were separated and stored at -80° C until oxidative stress analysis (within 4 months after blood sampling).

Measurements of oxidative status

Reactive oxygen metabolites (ROMs)

We estimated the plasma concentration of reactive oxygen metabolites (ROMs) using the d-ROMs test (Diacron International, Grosseto, Italy). This colorimetric assay measures circulating hydroperoxides (Alberti et al. 2000; Buonocore et al. 2000), which are intermediate oxidative damage molecules produced by peroxidation of diverse biomolecules, including lipids and proteins (Halliwell and Gutteridge 2007). The reaction of a dilution series of cumene hydroperoxide was highly linear (range: 0 to 4.5 μM, R^2 = 0.9996; physiological values in vertebrates). Analysis followed previously published protocols (Costantini et al. 2006). In short, the plasma (8 μl) was diluted with 200 μl of a solution containing acetate buffer (pH 4.8) and an aromatic alkyl-amine as chromogen. The samples were then incubated for 75 min at 37 °C. After incubation the absorbance was read with a Thermo Scientific Multiskan Spectrum spectrometer (ThermoFisher, Vantaa, Finland) at a wavelength of 505 nm.

The ROMs concentration was calculated by comparing the absorbance of the samples with a standard curve. Measurements were expressed as mM of H_2O_2 equivalents. Samples were run in duplicate. ROMs concentrations were highly repeatable within individuals (r = 0.87, $F_{106,107}$ = 14.909, P < 0.001) (Lessells and Boag 1987). The inter-assay coefficient of variation was 12.1%, and the intra-assay coefficient of variation was 4.3%.

Glutathione peroxidase activity (GPX)

The activity of glutathione peroxidase (GPX) in red blood cells was quantified using the Ransel assay (Randox Laboratories, Crumlin, UK) (Paglia and Valentine 1967). GPX is an antioxidant selenoenzyme that catalyzes the reduction of peroxides and hydroperoxides, using thiols as cofactors (Arthur 2000).

The assay was performed following the manufactures' protocol. In short, the samples were diluted 1: 40 with the provided dilution agent. 200 µl of reagent (glutathione 4 mmol l^{-1}; glutathione reductase ≥0.5 U l^{-1}; NADPH 0.34 mmol l^{-1}) was added to each plate well. 4 µl of the diluted samples and 8 µl cumene hydroperoxide were added and absorbance was read with a Thermo Scientific Multiskan Spectrum spectrometer (ThermoFisher, Vantaa, Finland) at 340 nm after one and three minutes. GPX activity was calculated using the formula reported in the manufacturer's instructions: [(Abs 1min-Abs 3min)/ 2]* 15'873]. GPX values were standardized by expressing them as units of GPX per mg proteins. The concentration of proteins in red blood cells was measured using the Bio-Rad Bradford Protein assay kit (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions. Repeatability of GPX activity within individuals was high (r = 0.98, $F_{327,328}$ = 91.797, P < 0.001) (Lessells and Boag 1987). The inter-assay coefficient of variation was

12.4% and the intra-assay coefficient of variation was 5%.

Red blood cells thiols (Thiols)

We measured thiol-containing compounds in red blood cells using the -SHp test (Diacron International, Grosseto, Italy) following Costantini et al. (2011). Thiols are compounds that contain a functional group composed of a sulphur atom and a hydrogen atom (-SH). Thioredoxin and glutathione are two of the major thiols that occur in animal cells. These molecules play important roles in antioxidant systems, acting as substrates for enzymes or directly neutralizing compounds, which cause oxidative damage, such as hydrogen peroxide (Dickinson and Forman 2002; Bindoli et al. 2008).

Red blood cells were diluted 1:200 with distilled water. Diluted red blood cells (12.5 µl) were added to a sulfate buffer (pH 7.6) and incubated at room temperature for 3 min. After incubation a baseline absorbance was read with a SpectraMax 340PC^{384} Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Then 5 µl of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added and incubated at room temperature for 5 min. The absorbance was then read again at 405 nm. Concentrations were calculated using a standard solution of L-cysteine (496 mM of -SH groups). Thiols measures were expressed as µmol of –SH groups per mg proteins. Repeatability of thiols measures within individuals was high (r = 0.98, $F_{333,334}$ = 95.101, $P < 0.001$) (Lessells and Boag 1987). The inter-assay coefficient of variation was 6.4% and the intra-assay coefficient of variation was 5.8%.

Plasma non-enzymatic antioxidant capacity (OXY)

We measured the non-enzymatic antioxidant barrier in the plasma by colorimetric

determination using the OXY- Adsorbent test (Diacron International, Grosseto, Italy). This assay quantifies the ability of the plasmatic antioxidant barrier, including exogenous and endogenous antioxidant compounds, to deal with the action of a powerful oxidant, hypochlorous acid (HOCl) (Carratelli et al. 2001).

The procedure was carried out according to previously published protocols (Costantini et al. 2006). Briefly, plasma samples (2 µl) were diluted 1:100 with distilled water. 2 µl diluted plasma was added to 200 µl of a titred HOCl solution and incubated at 37 °C for 10 min. After incubation, 2 µl of N,N-diethyl-*p*-phenilendiamine was added as a chromogen and the absorbance was read at a wavelength of 490 nm with a Thermo Scientific Multiskan Spectrum spectrometer (ThermoFisher, Vantaa, Finland). Measurements are expressed as mM of neutralized HOCl. Samples were run in duplicate. Repeatability within individuals was high (r = 0.89, $F_{282,283}$ = 17.973, P < 0.001) (Lessells and Boag 1987). The inter-assay coefficient of variation was 10.5% and the intra-assay coefficient of variation was 5.9%.

Statistical analyses

We used general linear mixed models (LMM) to test for effects of the pre-laying treatment, the post-hatching treatment, sex and their interactions on nestling oxidative stress biomarkers, fledging mass, tarsus length and growth rate. We also used a LMM to test for effects of the pre-laying treatment, sex and their interaction on hatching mass. Brood was included as a random factor to account for the non-independence of siblings due to shared genes and / or environmental factors.

Furthermore, to test whether any of the measured oxidative stress markers were associated with nestling growth and morphology, we added the oxidative stress

biomarkers as covariates in the LMMs described above. Since the stress biomarkers were not significantly correlated ($r < 0.127$, $P > 0.067$, in all cases) they were included simultaneously in the models.

The final models were obtained by removing the non-significant interactions and factors in a stepwise backward procedure, starting with the least significant interaction term. Brood (random effect), pre-laying treatment, post-hatching treatment and sex were retained in all final models. If interaction effects were significant, we performed post-hoc contrasts based on least squares means to determine which groups differed significantly from one another. Residuals of all models were checked for homoscedasticity and normality.

All oxidative stress measures were log transformed for the statistical analyses. Sample sizes differ slightly among oxidative stress marker analyses because not enough plasma was available to run all tests for all nestlings. Statistical analyses were performed in JMP 10 (Institute Inc. SAS 1989-2007).

Results

Blood oxidative status of nestlings

The pre-laying food supplementation affected the levels of oxidative damage (ROMs) in a sex-specific way (Table 1). Daughters had higher ROMs levels than sons if their mother had not received extra-food during the egg laying period (Posthoc contrast: $F_{1,175.5} = 7.569$, $P = 0.007$; Fig. 1a). No such sex-difference was observed in offspring of supplemented mothers (Posthoc contrast: $F_{1,183.7} = 2.678$, $P = 0.103$; Fig. 1a). Furthermore daughters of non-supplemented mothers had higher ROMs levels than daughters of supplemented mothers (Posthoc contrast: $F_{1,173.7} = 9.772$, $P = 0.002$; Fig. 1a), but no differences in ROMs levels in male offspring from supplemented and non-

supplemented mothers were observed (Posthoc contrast: $F_{1,146.2} = 0.048$, $P = 0.827$; Fig. 1a).

A similar sex difference in ROMs concentrations was found in relation to the post-hatching treatment (Table 1). Female nestlings had higher concentrations of ROMs than male nestlings when they did not receive any extra-food during the rearing period (Posthoc contrast: $F_{1,173.7} = 3.851$, $P = 0.051$; Fig. 1b), whereas males and females had similar concentrations of ROMs when they received extra-food during the rearing period (Posthoc contrast: $F_{1,185.6} = 1.161$, $P = 0.283$; Fig. 1b). Differences in ROMs concentrations between female nestlings that did or did not receive extra-food during the rearing period were non-significant (Posthoc contrast $F_{1,173.2} = 0.843$, $P = 0.359$; Fig. 1b). Similarly, differences in ROMs concentrations between male nestlings that did or did not receive extra-food during the rearing period were non-significant (Posthoc contrast: $F_{1,147.2} = 2.376$, $P = 0.125$; Fig. 1b). The two-way interaction between treatments and the three-way interaction between pre-laying treatment, post-hatching treatment and sex were not significant ($P > 0.176$ in all cases), and therefore removed from the final model.

Sex-differences were also found in the antioxidant defense markers analyzed. Female nestlings had significantly lower GPX activity than males (Table 1), and this effect was independent of the food treatments ($P > 0.373$ in all cases; see Electronic Supplementary Material (ESM) Fig. S1). Furthermore, there was a marginally significant interaction effect between nestling sex and the post-hatching food treatment on the concentrations of thiols (Table 1; Fig. 2b). Females tended to have lower thiols concentrations than males if they received no extra-food during the post-hatching period (Posthoc contrast: $F_{1,270} = 3.356$, $P = 0.068$; Fig. 2b), whereas no such sex difference was found in supplemented broods (Posthoc contrast: $F_{1,\,255.5} = 0.821$,

$P = 0.366$; Fig. 2b). All other interaction effects were non-significant ($P > 0.117$ in all cases: Fig. 2). We did not detect any effect of the treatments, sex or their interactions on OXY levels ($P > 0.303$ in all cases; Table 1; ESM Fig. S2).

Nestling growth and morphology

The pre-laying treatment had no significant effect on hatching mass (Table 2). Furthermore, pre- and post-hatching food treatments had no significant effect on nestling growth rate or fledging mass (Table 2). However, the pre-laying treatment had a marginal effect on tarsus length (Table 2), with offspring of non-supplemented mothers having slightly longer tarsi than offspring of supplemented mothers (mean ± SE: pre-F = 19.21 ± 0.05 mm; pre-NF = 19.34 ± 0.04 mm). There was no sex difference in hatching mass, but males grew faster and were larger and heavier than females shortly before fledging (Table 2). None of the interactions between treatments and between treatments and sex were significant ($P > 0.155$ in all cases), and therefore not retained in the final models.

GPX activity was significantly associated with growth rate, with nestlings that grew faster having lower GPX activity (Table 2). None of the other oxidative stress biomarkers were significantly associated with nestling growth rate, fledging mass or tarsus length (Table 2), and they were therefore not retained in the final models.

Discussion

Our study experimentally demonstrates that pre- and early postnatal nutritional conditions affect the oxidative status of nestling great tits in a sex-specific way. Daughters of non-supplemented mothers, and daughters which did not receive extra

food during the early postnatal period had higher oxidative damage than sons, while no differences between sons and daughters were observed when extra food was provided pre- or postnatally. These results show that nutritional conditions experienced early in life, and in particular resources transferred by the mother through the egg, play an important role in shaping the offspring's oxidative status.

These findings are in agreement with studies on laboratory rats showing that the amount of protein individuals acquire through the diet is crucial for an efficient antioxidant defense (Feoli et al. 2006; Blouet et al. 2007), and that maternal protein malnutrition impairs the functioning of the antioxidant machinery of the offspring (Bonatto et al. 2006). Interestingly, we observed no effect of the pre- or postnatal food supplementation on nestling growth, fledging mass or tarsus length. Given that non-supplemented females had higher level of oxidative damage, this suggests that when resources are limited, (female) nestlings maintained their investment in growth at the expense of oxidative defence.

A limitation of our study is that oxidative status biomarkers were measured only once during the nestling period (on day 9 post-hatching). Therefore, we could not assess how oxidative status changed over time and how the pre- and post-hatching treatments influence this dynamics. However previous studies on birds showed that the biomarkers of blood oxidative status used in this study have a significant within-individual repeatability over periods of days to years (Costantini et al. 2007; Saino et al. 2011; D.C. unpublished data).

Interestingly, prenatal conditions appear to be particularly important in influencing offspring oxidative damage. Indeed, the effect of the pre-laying food treatment on levels of oxidative damage was even more pronounced than the effect of the post-hatching food treatment. It suggests that mothers differentially allocated

resources into their eggs in response to the pre-laying treatment, and that these maternal resources had a strong and sex-specific effect on the offspring's oxidative status. We can exclude the possibility that the effect of the pre-laying food supplementation was due to a carry-over effect that influenced maternal provisioning after hatching, because all nestlings were cross-fostered and raised by foster parents. At the moment, we can only speculate what maternal egg component mediated the observed effects. In our experiment the food-supplementation consisted mainly of protein and fat (i.e. maggots). Thus supplemented mothers may have deposited more fat and / or proteins into their eggs, which may have boosted the ROS scavenging machinery through the enhancement of antioxidant synthesis and / or activity (Dringen et al. 1999; Lee et al. 2013). Indeed, food-supplemented females tended to lay heavier eggs.

Although we found evidence for sex differences in the antioxidant protection machinery of nestlings – females generally had a lower glutathione peroxidase (GPX) activity than males, and when no extra food was provided during the early postnatal period, they also tended to have lower thiols levels – no effect of the pre-laying treatment on these antioxidant defence components was observed. It indicates that egg composition affected an unmeasured aspect of the antioxidant machinery, which, in turn, influenced levels of oxidative damage in daughters. Alternatively, or in addition, egg composition may have affected the nestling's cell membrane composition (e.g., quantity of unsaturated fatty acids), which is known to affect the basal production of oxidative molecules (Hulbert et al. 2007).

Studies in mammals and birds often find that males are more susceptible to harsh environmental conditions than females (reviewed in Jones et al. 2009). It has been proposed that the larger body size, and the consequently higher need of

resources of males, is the main factor driving this pattern (Clutton-Brock et al. 1985). However, whereas in great tits males are slightly (2.3%) larger than females, it was the females that were more susceptible to pre- and postnatal food conditions. It might be that the larger body size placed male nestlings at a competitive advantage over access to food (see also Oddie 2000), causing the observed increase in oxidative damage in female nestlings when food was limited (e.g. because of increased begging (Noguera et al. 2010)). However, whereas a competitive advantage could explain why females had higher oxidative damage than males when receiving no extra food during the nestling period, it is unlikely to explain the sex differences in oxidative damage caused by the pre-laying food treatment. The sex-specific sensitivity to differential, food-mediated egg composition suggests that females are more constrained in the allocation of limited resources to the ROS defence machinery because of different investment priorities and life history strategies (e.g. a higher investment in immune defence (Norris and Evans 2000; Tschirren et al. 2003)), and / or sex-specific differences in embryonic development.

The higher levels of oxidative damage found in females in response to resource limitation is in contrast to the study of De Coster et al. (2012) who found that male great tit nestlings had higher levels of oxidative damage when exposed to ectoparasitic fleas. The opposite effects of these two important environmental factors (food availability (this study) vs. parasitism (De Coster et al. 2012)) on the oxidative status of male and female nestlings highlights the different sensitivities of the two sexes to environmental factors, with subsequent consequences on physiological mediators of variation in fitness (Noguera et al. 2012; Losdat et al. 2013). The higher sensitivity of female nestlings to food limitation early in life suggests that females might pay a higher price for the increasing mismatch between food availability and

demand due to climate change observed in many bird species (Thomas et al. 2001; Visser et al. 2003)

Generally, we found no strong relationships between the measured markers of oxidative damage or protection and nestling growth, fledging size or mass. However, there was a negative association between GPX activity and nestling growth rate, with nestlings, which grew faster, having lower GPX activity. Glutathione peroxidase activity relies on the presence of glutathione (GSH), which can also act as a scavenger and directly react with ROS (Bindoli et al. 2008). During increased physical activity, a depletion of GSH occurs, with consequent decrease in GPX activity (Leeuwenburgh and Ji 1995). Similarly, during growth nestlings undergo high metabolic activity, which can cause an increase of ROS production (Rollo et al. 1996). Hence, in order to avoid oxidative stress, a significant portion of GSH available in the blood may be depleted when reacting with ROS, and this may trigger a down-regulation of GPX synthesis. Alternatively, it might be that during growth a decrease in GPX activity occurs because GPX is directly used for ROS detoxification.

In conclusion our study shows that nutritional conditions experienced early in life, both before and shortly after birth, influence the oxidative status of great tit nestlings in a sex-specific way, with female nestlings being more sensitive to nutritional stress than males. The physiological disadvantages of females are probably not linked to higher resource requirements, since female great tits are smaller than males. It indicates that sex-specific allocation priorities due to different life history strategies caused the observed patterns.

Acknowledgements

We thank Martina Keller, Kathrin Näpflin and Jamal Hanafi for field assistance and help in the lab and two anonymous reviewers for comments on the manuscript. The study was financially supported by the Swiss National Science Foundation (grant PP00P3_128386 B.T.). All procedures were conducted under licence from the Veterinary Office of the Canton of Zurich (195/2010) and the Federal Office for the Environment (324).

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Figures legends

Fig. 1 Effect of the pre-laying (a) and post-hatching (b) food treatments on plasma oxidative damage (ROMs) in female (filled circles) and male (empty circles) great tit nestlings. The *x*-axis indicates food-supplementation (F) and not-supplementation (NF) treatments. Means ± 1 S.E. are shown

Fig. 2 Effect of the pre-laying (a) and post-hatching (b) food treatments on red blood cell thiols in female (filled circles) and male (empty circles) great tit nestlings. The *x*-axis indicates food-supplementation (F) and not-supplementation (NF) treatments. Means ± 1 S.E. are shown

Table 1

Effects of the food treatments and sex on plasma oxidative damage (ROMs, mM H_2O_2 equivalents; N = 226), glutathione peroxidase activity (GPX, U GPX mg^{-1} proteins; N = 328), red blood cells thiols (Thiols, µmol of -SH groups mg^{-1} proteins; N=334), plasma non-enzymatic antioxidant capacity (OXY, mM HOCl neutralized; N = 300). Brood was included as a random factor in all analyses. The variance explained by brood is presented. * $P < 0.05$

		F	*df*	*P*
ROMs				
	Pre-laying treatment	3.460	1,95.8	0.066
	Post-hatching treatment	0.008	1,95.7	0.931
	Sex	0.236	1,180.2	0.628
	Sex × Pre-laying treatment	9.240	1,177.7	0.003*
	Sex × Post-hatching treatment	5.038	1,177.3	0.026*
	Brood (variance explained: 45.1%)			
GPX				
	Pre-laying treatment	0.064	1,112.7	0.801
	Post-hatching treatment	2.326	1,112.7	0.130
	Sex	10.414	1,241.1	0.001*
	Brood (variance explained: 63.5%)			
Thiols				
	Pre-laying treatment	2.572	1, 110.8	0.116
	Post-hatching treatment	0.001	1,110.8	0.913
	Sex	0.517	1,263.2	0.473
	Sex × Post-hatching treatment	3.910	1,262.6	0.049*
	Brood (variance explained: 45.8%)			
OXY				
	Pre-laying treatment	0.087	1,109	0.769

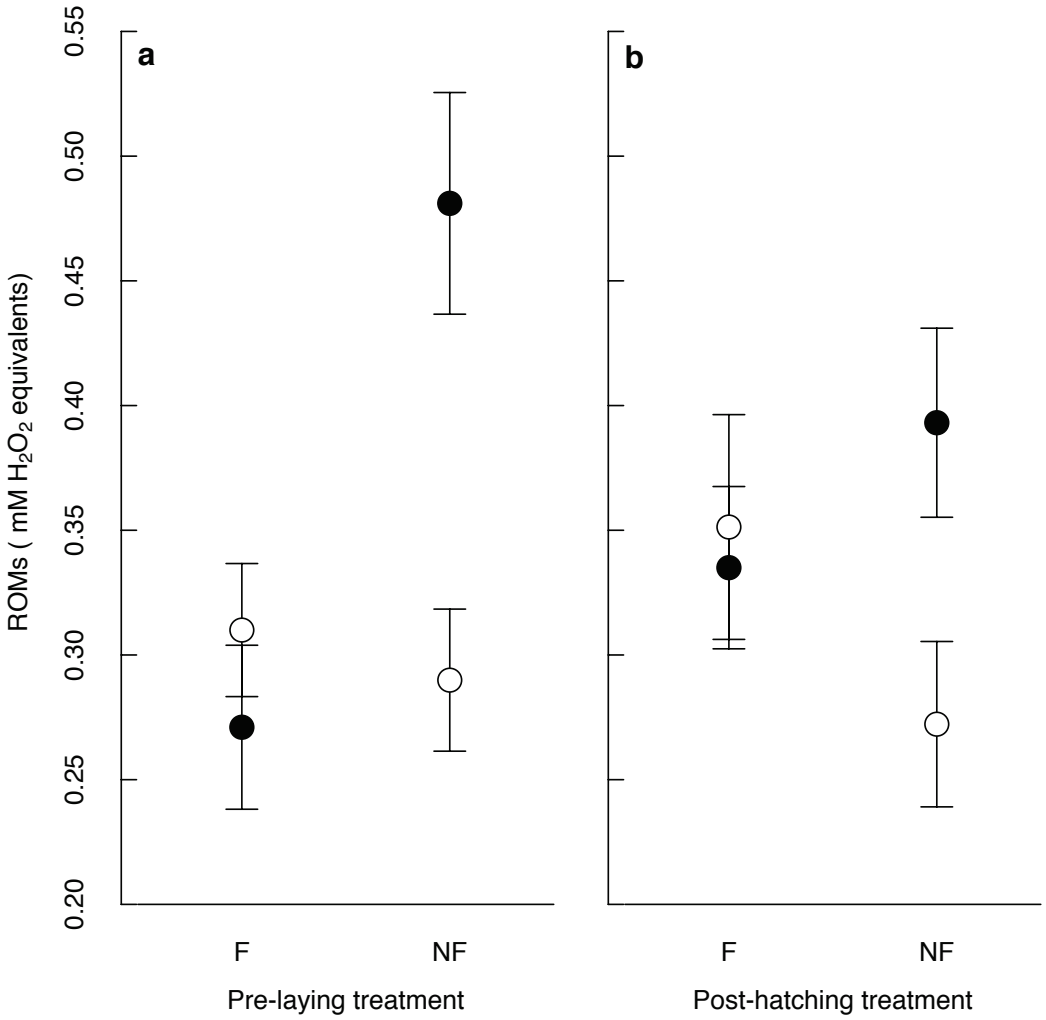
Post-hatching treatment	1.899	1.109.3	0.171
Sex	0.265.3	1,265.3	0.633
Brood (variance explained: 28.9%)			

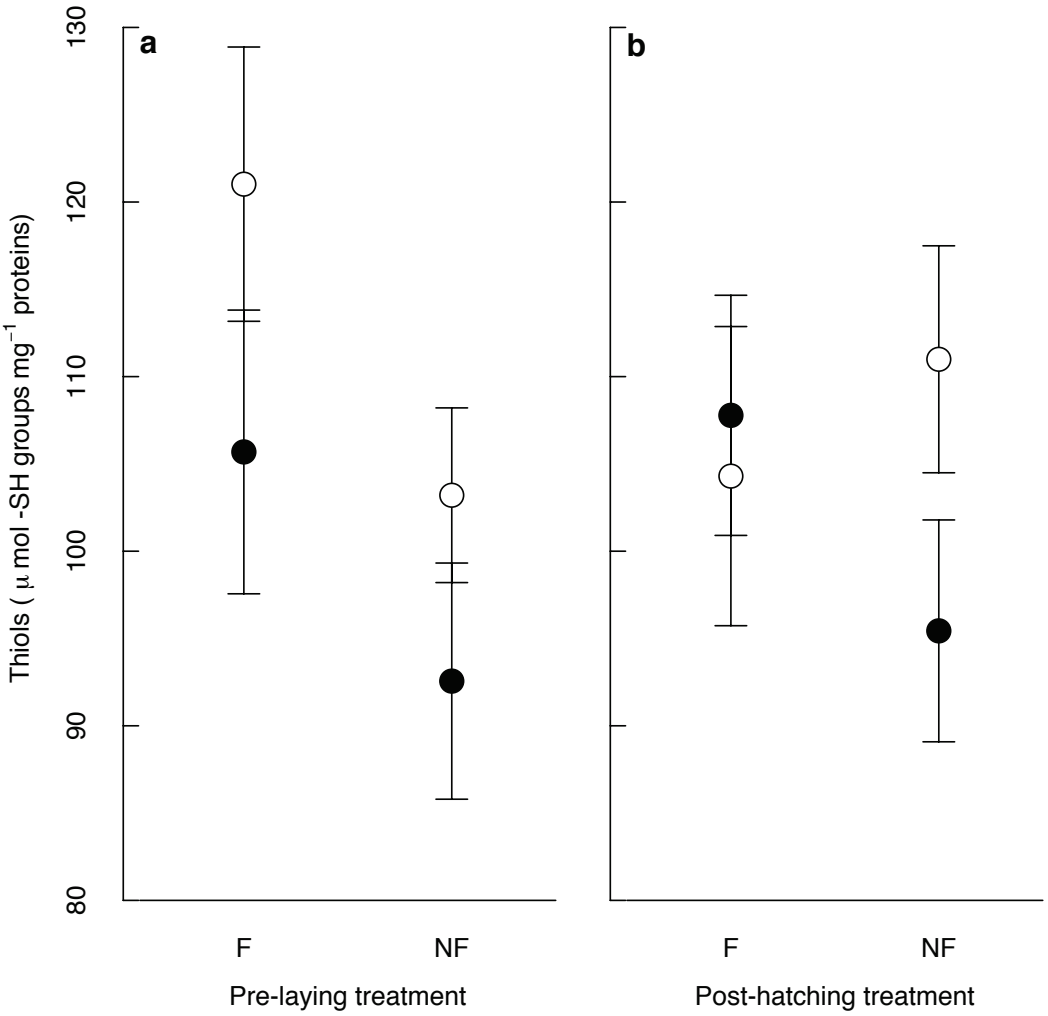
Table 2

Effects of the food treatments, sex and oxidative biomarkers (ROMs, mM H_2O_2 equivalent; GPX, U GPX mg^{-1} proteins; Thiols, µmol of -SH groups mg^{-1} proteins; OXY, mM HOCl neutralized) on body mass at hatching (N = 756), body mass on day 15 (N = 517), tarsus length on day 15 (N = 517), and growth rate between day 3 and day 9 (N = 651). Brood was included as a random factor in all analyses. The variance explained by brood is presented. Terms not retained in the final models are highlighted in italic. Values of terms not retained in the models are those prior to removal. * P < 0.05

		F	*df*	*P*
Hatching mass (g)				
	Pre-laying treatment	2.252	1,121.8	0.136
	Sex	1.501	1,608.4	0.221
	Brood (variance explained: 58.8%)			
Fledging mass (g)				
	Pre-laying treatment	0.139	1,90.1	0.709
	Post-hatching treatment	1.787	1,90.1	0.185
	Sex	56.785	1,439.5	<0.001*
	ROMs	2.426	1,162.9	0.121
	GPX	2.216	1,166.7	0.138
	Thiols	1.774	1,152.8	0.185
	OXY	0.719	1,130.4	0.399
	Brood (variance explained: 64.4%)			
Tarsus length (mm)				
	Pre-laying treatment	3.144	1,90.6	0.079
	Post-hatching treatment	0.003	1,90.6	0.954
	Sex	79.883	1,461.1	0.001*
	ROMs	1.397	1,177.7	0.239
	GPX	0.027	1,128.8	0.875
	Thiols	0.069	1,149.3	0.793

	OXY	2.888	1,236.1	0.090
	Brood (variance explained: 39.7%)			
Growth rate (g day^{-1})				
	Pre-laying treatment	0.588	1, 108.9	0.445
	Post-hatching treatment	0.008	1,109.8	0.931
	Sex	7.558	1,234.9	0.003*
	ROMs	0.002	1, 163.2	0.988
	GPX	5.001	1,311.6	0.026*
	Thiols	1.258	1,236.5	0.289
	OXY	0.779	1,220.4	0.378
	Brood (variance explained: 65.9%)			


a
b
ROMs (mM H_2O_2 equivalents)
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
F
NF
F
NF
Pre-laying treatment
Post-hatching treatment


a
b
Thiols (µ mol -SH groups mg^{-1} proteins)
80
90
100
110
120
130
F
NF
F
NF
Pre-laying treatment
Post-hatching treatment